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Effects of Chlordiazepoxide, Flumazenil and DMCM on Plasma Catecholamine and Corticosterone Concentrations in Rats¹

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DE BOER, S. F., J. VAN DER GUGTEN AND J. L. SLANGEN. *Effects of chlordiazepoxide, flumazenil and DMCM on plasma catecholamine and corticosterone concentrations in rats.* PHARMACOL BIOCHEM BEHAV 38(1) 13–19, 1991.—The effects of drugs representing three classes of benzodiazepine (BDZ) receptor-acting agents on circulating corticosterone (CS), noradrenaline (NA) and adrenaline (A) were examined in unstressed rats. Intragastric administration of a single-dose of the inverse agonist 3-carbomethoxy-4-ethyl-6,7-dimethoxy- β -carboline (DMCM; 10 mg/kg) evoked 15-, 4- and 1.5-fold increases in plasma CS, A and NA, respectively, as compared to control values. The DMCM-induced CS, A and NA rises were completely blocked by combined treatment with the BDZ antagonist flumazenil (Ro 15-1788; 20 mg/kg). Flumazenil given alone did not affect plasma hormone levels. Administration (either intragastrically or intraperitoneally) of a single-dose of the BDZ agonist chlordiazepoxide (CDP; 20 mg/kg) produced a 10- to 15-fold increase in plasma CS but caused no change in plasma NA and A contents. Pretreatment with flumazenil blocked the CDP-elicited release of CS. The findings indicate that the CNS mechanisms controlling pituitary-adrenocortical and sympatho-adrenal outflow under basal conditions are functionally linked to central-type BDZ receptor system(s). Drugs with agonist or inverse-agonist actions at these receptor sites can be differentiated from each other by their distinct effects on plasma NA and A, but not CS, release.

Noradrenaline	Adrenaline	Corticosterone	Chlordiazepoxide	Flumazenil	DMCM	BDZ receptor ligands
Rat						

STRESSFUL or anxiety-provoking environmental events generally activate the pituitary-adrenocortical axis and the sympathetic-adrenomedullary system resulting in elevated blood plasma concentrations of the glucocorticoid corticosterone (CS) and of the catecholamines (CAs) noradrenaline (NA) and adrenaline (A) (1,7). In experimental animals as well as in man, these neuroendocrine stress responses can be mimicked by administration of benzodiazepine (BDZ) receptor ligands with so-called inverse agonist (i.e., anxiogenic) intrinsic activity such as the β -carbolines DMCM, FG 7142 or β -CCE (6, 15, 25, 26, 29). In accordance with the anxiogenic properties of these compounds is their ability to enhance the stress-elicited increases in CS release (26).

On the other hand, numerous reports have demonstrated that BDZ receptor ligands with agonist (i.e., anxiolytic) intrinsic actions like the benzodiazepines chlordiazepoxide (CDP) or diazepam, can prevent or oppose the stress-induced activation of pituitary-adrenocortical (10, 11, 16–20, 22, 27, 29, 31) and sympathetic-adrenomedullary systems (4,34). In addition to anxiolytic and anxiogenic BDZ-ligands, a third class of substances (i.e., the BDZ-receptor antagonists) appears to exert little intrinsic effect itself at the central BDZ-receptor, but is able to effectively antag-

onize the pharmacological effects of both the agonists and the inverse agonists. Indeed, the prototypical BDZ receptor antagonist, flumazenil (Ro 15–1788), was shown to block the stress-like elevations in plasma CS, NA and A concentrations induced by the inverse agonist β -CCE (25) and to prevent the inhibition of stress-elevated CS contents by the BDZ agonist diazepam (2,8). Based on these findings, it has been proposed that BDZ-agonists with anxiolytic or antistress activity are well characterized by their potency to inhibit stress-induced elevations in plasma CS and/or CA concentrations, while the ability to enhance these hormone levels in unstressed rats is a reflection of anxiogenic or stress-inducing properties typical of BDZ-inverse agonists (13, 19, 29).

Logically, it would be expected that BDZs and other agents possessing anxiolytic activity would only reduce the activity in stress-activated neuroendocrine systems and not elevate the levels of stress hormones under basal conditions. However, this appears not to be the case since anxiolytic doses of BDZs also elevate (dose-dependently) CS concentrations when administered acutely to unstressed rats (16, 20, 22, 27, 31). These anxiogenic or pro-stress effects are generally believed to arise from the sedative/ataxic actions of BDZs (22, 27, 31), although such a relationship

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has been questioned (11). Hence, it seems that the enhancement of plasma CS levels in unstressed rats is not clearly related to a specific action at the BDZ receptor since it is produced by agonists as well as by inverse agonists of this receptor. However, it has not been established yet whether this effect of BDZ agonists, like that of the inverse agonists (25), is mediated by central BDZ receptors. Furthermore, it is not known whether agonists of the BDZ receptor also have the inverse agonist-like property to cause an increase in basal plasma NA and/or A concentrations.

Therefore, the first aim of the present study was to assess the effects of a prototypical representative of the three different groups of BDZ receptor ligands, i.e., the agonist chlordiazepoxide, the antagonist flumazenil and the inverse-agonist DMCM, on plasma CS and CA concentrations in unstressed rats. The second goal was to determine whether the actions of CDP and DMCM can be blocked by pretreatment with flumazenil, in order to establish if the effects of these drugs are mediated through central BDZ-receptors. Finally, in order to minimize the nondrug-related stress associated with common methods of drug administration (e.g., handling, IP or SC injection, orogastric intubation), an intragastric cannulation method was employed and validated in this study.

METHOD

Animals and Housing

Male Wistar rats (Harlan-CPB, Zeist, The Netherlands) weighing 250–275 g on their arrival in the laboratory were used as subjects. They were housed individually in clear Plexiglas cages (25 × 25 × 30 cm) on a layer of woodshavings. For at least two weeks prior to surgery, cages were placed in a room under conditions of constant temperature ($21 \pm 2^\circ\text{C}$) and a fixed 12-h light/dark photoperiod (lights on at 07:00 a.m.). Subjects were in full view, sound and smell of each other, had free access to food and water at all times and were handled daily for weighing purposes.

Surgery and Blood Sampling

Under Hypnorm anesthesia (10 mg/kg fluanisone and 0.2 mg/kg fentanyl) and premedicated with atropine (1 mg/kg) and diazepam (5 mg/kg), animals were provided with a silastic cannula (i.d. 0.5 mm; o.d. 0.9 mm; Dow Corning, USA) into the entrance of the right atrium via an external jugular venotomy according to the techniques basically described by Steffens (28). This cannula allows frequent withdrawal of small amounts of blood without disturbing the animal either behaviorally or physiologically (28,35). Animals were also provided with a silicon cannula (i.d. 0.8 mm; o.d. 1.4 mm) into the antrum wall of the stomach. The outer ends of the cannulae were extended subcutaneously to emerge at the top of the head and anchored to the skull (30). This indwelling catheter allows intragastric drug administration in the freely behaving and undisturbed rat. After surgery, the rats were allowed to recover for at least one week before the start of the experiments. During this period, animals were accustomed to the blood sampling procedure.

At least ninety minutes before the start of an experiment, the indwelling cannulae were extended by polyethylene tubes (length 0.5 m; o.d. 1.45 mm; i.d. 0.75 mm) allowing blood sampling and intragastric drug infusion. Blood samples of 0.35 ml for the determination of NA, A and CS concentrations were taken at $t = 0, 15, 30, 60, 120$ and 180 min. Immediately after each blood sampling an equal volume of heparinized (12.5 IU/ml) blood, freshly obtained from a cannulated donor rat, was transfused through the catheter. At the end of the experiment, the indwelling part of the heart cannula was filled with 0.9% (wt./vol) NaCl containing 500 IU heparin/ml plus 60% polyvinylpyrrolidone

(Merck, Darmstadt, FRG). The indwelling part of the stomach catheter was filled with 0.9% saline.

Drugs

Chlordiazepoxide hydrochloride (Hoffmann-La Roche, Basel, Switzerland) was dissolved in physiological (0.9%) saline. The vehicle consisted of an acidified (HCl) saline solution with a similar acidity as the respective CDP solution (pH 3.1). In contrast to the acidified saline solution, the CDP solution also has a considerable buffering capacity. Therefore, two additional vehicles consisting of 0.1 M sodium-citrate/HCl buffer with a pH of 3.0 and 5.0, respectively, that had an approximately similar buffering capacity as the CDP solution, were used in Experiment 1. Flumazenil (Ro 15-1788; Hoffmann-La Roche, Basel, Switzerland) was suspended in a vehicle consisting of distilled water to which Tween 80 (2 drops/10 ml) was added. DMCM (methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate; Schering, Berlin, FRG) was dissolved in an acidified (HCl) saline solution. All solutions were freshly prepared before every test. In case of intraperitoneal (IP) injections, drug and vehicles were administered in a volume of 1 ml/kg. In case of intragastric administration a volume of 2 ml/kg was used.

Experimental Procedures

All experiments were performed in the light period between 09:00 and 13:00.

Experiment 1: Comparison of the Effects of Intraperitoneal and Intragastric CDP Administration on Plasma CS and CA Content

Nine groups of 6 animals each were used in this experiment. Rats in the CONTROL group did not receive any drug or vehicle treatment and were left undisturbed in their home cages during the whole 120-min sampling period. Rats in the HANDLING condition were gently picked up from their home cages immediately after taking the first, baseline, blood sample at $t = 0$ min, held (± 5 s) in the hand as if an IP injection was given and then returned to their home cages. Subjects in the IP-treatment groups were injected intraperitoneally with either acidified SALINE, buffer pH = 3, buffer pH = 5 or 20 mg/kg CDP. Subjects in the intragastric-treatment groups were not handled but immediately after taking the first blood sample, CDP (20 mg/kg) or vehicle (acidified SALINE and buffer pH = 3) solutions were slowly (10 s) infused via the intragastric cannula. Blood samples were taken according to the time schedule described above and indicated in the figures.

Experiment 2: Effects of CDP, DMCM and Flumazenil on Plasma CA and CS Concentrations

Twenty-three rats were randomly assigned to one of three conditions. All rats received two treatments using a balanced repeated-measures within-subject design with an intertest interval of one week. Each treatment consisted of two subsequent intragastric infusions. One group ($n = 6$) received vehicle/saline and flumazenil/saline treatment. The second group ($n = 6$) received vehicle/CDP and flumazenil/CDP, whereas rats in the third group ($n = 11$) received the treatments vehicle/DMCM and flumazenil/DMCM. Flumazenil and CDP were administered in a dosage of 20 mg/kg (66.0 and 59.5 $\mu\text{mol/kg}$, respectively); DMCM was administered in a dosage of 10 mg/kg (30.8 $\mu\text{mol/kg}$). Drug and vehicle solutions were infused via the intragastric cannula. Blood

samples were taken at the times indicated in the figures.

Chemical Determinations

Blood samples were immediately transferred to ice-cooled centrifuge tubes containing 10 μ l heparin solution (500 IU/ml). For the determination of plasma catecholamine contents, an aliquot of 250 μ l transferred blood was rapidly pipetted into chilled tubes containing 10 μ l of a solution of 25 mg/ml disodium EDTA and 27.5 mg/ml reduced glutathione in order to prevent CA degradation. The remaining 100 μ l blood was used for the CS assays and for the BDZ-receptor binding assays. After centrifugation ($4000 \times g$ for 10 min at 4°C), supernatants were removed and stored at -30°C until assayed.

The concentrations of NA and A were measured in duplicate in 20 μ l perchloric acid-deproteinized plasma according to a radioenzymatic COMT-procedure (33). In short, the CAs were converted into their [^3H]-methoxy derivatives by incubation with S-adenosyl-L-[methyl- ^3H]methionine (80 Ci/mmol; NEN Chemicals) in the presence of catechol-O-methyltransferase. Labeled products were isolated by organic extraction and paper chromatography. After elution of the labeled products, activity was counted in a liquid scintillation analyzer (Philips, The Netherlands). CA concentrations were calculated from net DPM values for samples and internal standards and expressed as pg/ml. The intra- and interassay variabilities were less than 10% and 15%, respectively. The sensitivity of the assay was 1 pg for both NA and A.

Plasma CS concentrations were determined in duplicate according to a competitive protein-binding method (24). Corticosterone was extracted with dichloromethane from 25 μ l samples of plasma and the dry residue was incubated with a corticosteroid-binding globulin tracer solution {0.1% plasma from adrenalectomized female rats containing [1,2- ^3H]-corticosterone (40–50 Ci/mmol; NEN Chemicals) as tracer}. Unbound steroid was removed using dextran-coated charcoal. Standard CS was supplied by Sigma. The intra- and interassay coefficients of variation were less than 10%. Fifty percent displacement of tracer steroid was obtained at a concentration of 20 ± 2 $\mu\text{g/dl}$.

Benzodiazepine activity in plasma was measured according to a radioreceptor procedure (14), modified with respect to radioligand and receptor preparation (23) and adapted to small plasma samples. BDZ ligands were extracted from 50 μ l samples in 175 μ l ethylacetate. Flumazenil containing plasma was diluted 20 times before extractions. Extract fractions were evaporated and incubated with 900 μ l bovine frontal cortex homogenate and 25 nCi [methyl- ^3H]flunitrazepam (60 Ci/nmol; NEN Chemicals) at 4°C for 60 min. Unbound benzodiazepine was removed by rapid filtration through Whatman GF-B filters. CDP standards were used to calculate CDP equivalent levels in plasma. Plasma CDP equivalent concentrations of 41.5 nmol/ml (for CDP and DMCM) or 830 nmol/ml (for flumazenil) resulted in 50% inhibition of specific radioligand binding.

Statistical Analyses

The response-time patterns of the plasma constituents in Experiment 1 were evaluated by use of two-way analyses of variance (ANOVA) with drug/vehicle-treatment as between-subject factor and sampling time as repeated-measures within-subject factor. The profiles of hormonal changes in Experiment 2 were analyzed using a three-way ANOVA with experimental group as between-subject factor (3 levels), treatment as within-subject factor one (2 levels) and sampling time as repeated measures within-subject factor two (6 levels). The multivariate model was used

for the repeated-measures factor (5). Further analyses were made by paired Student *t*-tests (within-group comparisons) or by Duncan's new multiple range test (between-group comparisons) to determine the source of the detected significance in the ANOVA's (3). The criterion of significance was set at $p < 0.05$.

RESULTS

Experiment 1

As shown in Fig. 1, the levels of plasma CS in uninjected and undisturbed control rats were low (0.8–2.3 $\mu\text{g/dl}$) and were found not to vary significantly at any time point, indicating that the method of blood collection was stress-free. Handling resulted in a small plasma CS increase, which was significant (see legend to figures for the results of ANOVA) at $t = 15$ and 30 min and returned to basal level at 60 min. Intraperitoneal injection of acidified saline or buffer (pH = 5) solution produced a similar pattern of changes in plasma CS levels as compared to that of the handled group of rats (Fig. 1A). The plasma CS values at 15 and 30 min after injection were elevated approximately 4- to 5-fold as compared to controls or preinjection levels, but were not significantly higher than the corresponding handling-elevated values. In contrast, following IP administration of a buffer solution with pH = 3, the increases in CS were evidently more pronounced and longer lasting than the increments after either handling, saline-injection or buffer pH = 5 administration. A single-dose administration of CDP produced a marked, long-term increase (10- to 15-fold) in plasma CS concentration. The CDP-induced increases at $t = 15$ and 30 min did not differ from the corresponding pH 3 buffer-treated values but were significantly higher as compared to the values of the other groups of rats.

Unlike after IP injection, plasma CS levels did not change over time following intragastrically applied acidified saline or buffer solution with pH = 3 (Fig. 1B). However, intragastric administration of 20 mg/kg CDP resulted in significant CS elevations at $t = 30$, 60 and 120 min. The time courses of the CDP-induced plasma CS changes following the two methods of drug administration were compared using a two-way ANOVA with method of drug administration as between-subject factor and sampling time as repeated measures within-subject factor. In addition to significant main effects of method, $F(1,10) = 60.8$, and sampling time, $F(4,7) = 137$, a significant method \times time interaction effect was found, $F(4,7) = 14.8$. This indicates that the profile of CDP-induced changes in CS contents were different between the two methods of drug delivery. The CS values at $t = 15$, 30 and 60 min after IP administered CDP were significantly higher as compared to the corresponding values following intragastrically applied CDP. These differences in CS levels were not due to differences in plasma CDP concentrations, since the time course of plasma CDP levels following intraperitoneal and intragastric administration of 20 mg/kg CDP were grossly similar (Fig. 2). Although the increase in CDP levels over the sampling period tended to be somewhat higher in the IP dosed group as compared to the intragastrically treated group of animals, this difference only reached significance at $t = 15$ min after drug administration (see legend to Fig. 2 for results of ANOVA).

Plasma NA and A concentrations did not vary significantly over the sampling period in any of the different treatment groups of rats. In all groups of rats plasma NA fluctuated within narrow limits around a mean of 456 ± 41 pg/ml. Circulating A fluctuated closely around a mean of 235 ± 22 pg/ml.

Experiment 2

As illustrated in Fig. 3, intragastric administration of the BDZ

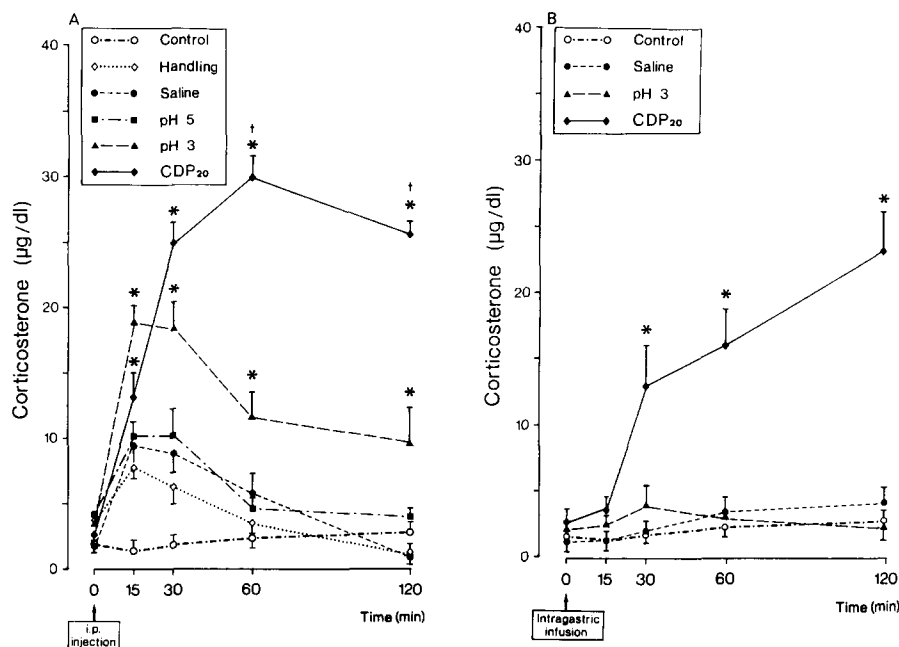


FIG. 1. Time course of plasma corticosterone (CS) levels following 20 mg/kg chlordiazepoxide (CDP) and various vehicle solutions (acidified saline, buffer pH=3 and buffer pH=5) administered either by intraperitoneal injection (A) or by intragastric infusion via an indwelling stomach catheter (B). Also illustrated are the CS levels of control rats left undisturbed in their home cages (A and B), and of handled rats (A). Data represent mean \pm SEM from 6 animals per treatment-condition. Results of ANOVA on the data presented in A are as follows: $F(\text{treatment})(5,30) = 68.9$, $p < 0.001$, $F(\text{time})(4,27) = 125$, $p < 0.001$, $F(\text{treatment} \times \text{time})(20,120) = 6.71$, $p < 0.001$. Results from ANOVA on data in B: $F(\text{treatment})(3,20) = 73.7$, $p < 0.001$, $F(\text{time})(4,17) = 39.3$, $p < 0.001$, $F(\text{treatment} \times \text{time})(12,57) = 2.77$, $p < 0.01$. *Significantly different from corresponding control value or from preinjection ($t=0$ min) value. †Significantly different from buffer pH=3-treated group values at similar time points.

inverse agonist DMCM (10 mg/kg) resulted in rapid, time-related increases in plasma CS, A and NA concentrations. Maximal CS levels (15-fold increase over baseline) were achieved between 30 and 60 min after DMCM administration, whereafter they declined and returned at $t=180$ min to basal values. Plasma A, which is released almost exclusively from the adrenal medullae, was elevated approximately 3.5- to 4-fold as compared to vehicle/saline controls or pretreatment values. The DMCM-induced increases in plasma A returned towards basal level by 60 min (Fig. 3B). Plasma NA, which is primarily released from sympathetic nerve endings, increased approximately 1.5-fold at 15 min following DMCM administration and returned to basal level by 30 min (Fig. 3B). The DMCM-induced plasma CS, A and NA elevations were completely blocked after pretreatment with the BDZ antagonist flumazenil (20 mg/kg). Flumazenil when administered alone, did not affect plasma CS and CA concentrations.

Intragastric administration of CDP (20 mg/kg) resulted in a profile of CS elevations similar to that observed in Experiment 1. Accordingly, the 20 mg/kg dose of CDP did not affect plasma NA and A concentrations. The CDP-evoked rise in CS differed from the DMCM-induced CS elevation both in magnitude (lower) and temporal pattern (longer lasting). However, like the DMCM-induced response, the CDP-elicited rise in CS was completely antagonized following combined treatment with flumazenil (20 mg/kg).

Table 1 shows the dynamics of plasma BDZ receptor activity (expressed as CDP equivalents) following single-dose administra-

tion of CDP, flumazenil and DMCM. All three BDZ ligands are rapidly absorbed after intragastric administration, reaching peak levels in about 15–30 min. Thereafter, BDZ activity declined rapidly in the case of flumazenil and DMCM, but remained elevated in the case of CDP treatment.

DISCUSSION

The results demonstrate that acute enteral (i.e., intragastric) or parenteral (i.e., intraperitoneal) administration of CDP to rats elicits a significant increase in plasma CS concentrations without any significant effect on circulating levels of NA and A. Clearly, the nondrug-related stress associated with the intraperitoneal injection procedure (i.e., handling, visceral disturbance arising from unphysiological physicochemical properties of the drug solution) contributes significantly to the IP CDP-induced rise in CS. Similar nondrug-related stress effects are absent when CDP is administered via an intragastric cannula, indicating that this method of drug administration can be used successfully to study the actions of drugs on neuroendocrine parameters in undisturbed freely behaving rats. It has previously been shown that handling and intraperitoneal injection of saline results in an enhancement of plasma CS levels in rats (21,27). Furthermore, our CDP effects on plasma CS are in general agreement with several other studies (17, 20, 31) demonstrating elevation of CS triggered by acute intraperitoneal or subcutaneous BDZ agonist administration including CDP. Since the increases in basal CS concentration are only

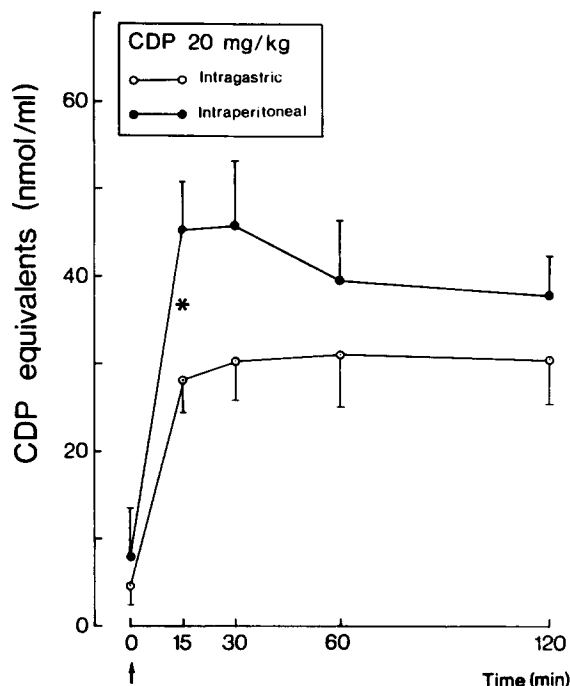


FIG. 2. Time course of plasma CDP concentrations (as determined by benzodiazepine radioreceptor assay) following acute intraperitoneal (closed symbols) or intragastric (open symbols) administration of 20 mg/kg CDP to unstressed rats. Data are expressed as mean \pm SEM for groups of 6 animals. ANOVA revealed a tendency ($p=0.06$) for the treatment factor to be significant, $F(1,10)=4.08$, and a significant main effect of time, $F(4,7)=27.1$. The interaction treatment \times time was not significant. Asterisk indicates a significant difference between the two group values (Duncan's new multiple range test).

seen with the use of medium to high, sedative, doses of CDP and not with low doses, it has been suggested that these effects are related to the behaviorally depressant action of CDP (18,31). The lack of significant increases in plasma NA and A contents following handling and intraperitoneal vehicle injection is most likely due to the timing of sample collection. In previous reports we have demonstrated that handling produced rapid but transient (return to basal values within 15 min) elevations in NA and A (9). Accordingly, a rapid CDP-induced rise and fall in plasma CA concentration within 15 min may have been missed in this study.

It has been established previously that BDZ agonists influence CS secretion through a central (i.e., brain) rather than peripheral (i.e., pituitary or adrenal gland) site of action (19,20). To date, the exact receptor mechanism (i.e., central-type or peripheral-type BDZ receptor) has not been elucidated. Results from the present study, however, show that coadministration of the central-type BDZ receptor antagonist flumazenil completely prevented the CDP-induced elevation of CS secretion. This is clear evidence that the effects of CDP upon the pituitary-adrenocortical axis in unstressed rats are mediated by central-type BDZ receptors. Since pituitary-adrenocortical output is regulated by a number of CNS pathways (1,12) which are all subserved by BDZ receptors (36), the specific loci of this CDP-mediated action remain unknown.

Intragastric administration of the BDZ inverse agonist DMCM produces a marked rise in rat CS levels. But, in contrast to CDP, DMCM causes substantial increases in plasma NA and A concentrations, indicating sympatho-adrenal activation. The greater adrenaline than noradrenaline release suggests that the adrenomedullary component of the sympatho-adrenal system is activated more by DMCM than the neurosympathetic branch. Given the anxiogenic properties of DMCM, this result reinforces the view (7,9) that plasma A concentration is a hormonal index of emotional stress, whereas plasma NA contents is more related to physical activity. More or less similar stress-like effects of BDZ inverse agonists have previously been reported (6, 25, 26, 29, 32).

The present finding that flumazenil effectively blocks the DMCM-elicited CS and CA response, is consistent with another study (25) showing that the anxiogenic effects of inverse agonist β -carboline are mediated through central-type BDZ receptors.

TABLE 1
DYNAMICS OF PLASMA BENZODIAZEPINE RECEPTOR BINDING ACTIVITY FOLLOWING INTRAGASTRIC ADMINISTRATION OF CDP, FLUMAZENIL AND DMCM

	Time After Drug Administration (min)					
	0	15	30	60	120	180
CDP (20 mg/kg)	4.8 \pm 0.8	27.9 \pm 2.8*	30.1 \pm 3.6*	31.3 \pm 3.6*	31.3 \pm 5.2*	26.7 \pm 4.8*
Flumazenil (20 mg/kg)	8.7 \pm 23.7	323.6 \pm 145.5*†	341.1 \pm 127.7*†	126.2 \pm 55.2*†	31.8 \pm 14.0*	2.9 \pm 9.8*‡
DMCM (10 mg/kg)	2.4 \pm 1.7	285.7 \pm 46.3*†	244.1 \pm 77.5*†	126.0 \pm 41.6*†	46.1 \pm 11.1*	57.8 \pm 13.8*

Values (mean \pm SEM) are expressed as CDP equivalents (μ mol/l) of benzodiazepine plasma levels as determined by radioreceptor assay. $n=6$ for each drug-treatment group.

Two-way ANOVA on the values revealed significant effects of the main factor time, $F(5,11)=13.5$, $p<0.001$, and a significant treatment \times time interaction effect, $F(10,24)=3.87$, $p<0.01$. Results of subsequent analyses are indicated by the following symbols: * $p<0.05$ as compared to the basal, time 0, value of the respective group. † $p<0.05$ as compared to corresponding CDP group value. ‡ $p<0.05$ as compared to corresponding flumazenil group value.

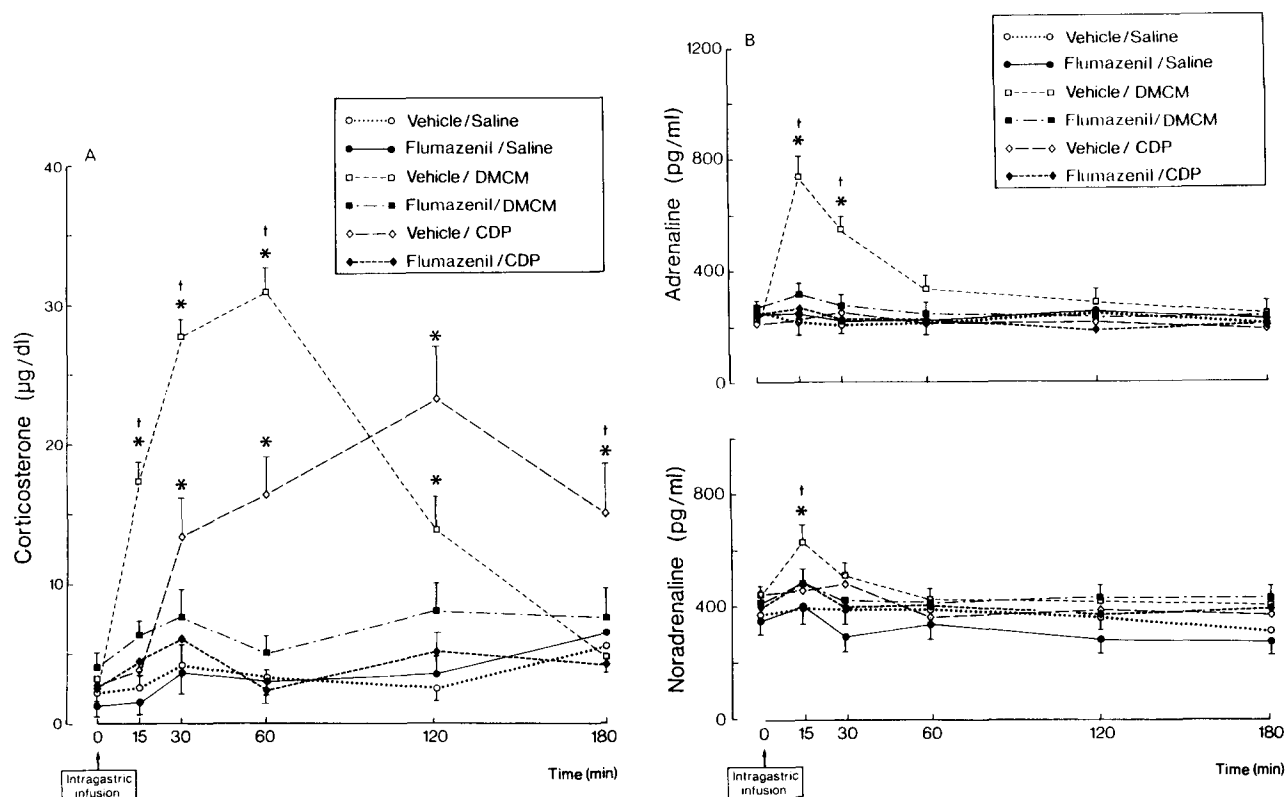


FIG. 3. Time course of plasma corticosterone (A), noradrenaline and adrenaline (B) levels after intragastric administration of CDP (20 mg/kg; $n = 6$), DMCM (10 mg/kg; $n = 11$) or acidified saline (2 ml/kg) combined with either flumazenil (20 mg/kg) or the flumazenil vehicle (2 ml/kg). Data represent mean \pm SEM. For the corticosterone and adrenaline data, three-way ANOVA revealed in addition to significant main and two-way interaction effects of drug condition, flumazenil treatment and sampling time, a significant three-way interaction [CS: $F(10,34) = 10.4$, $p < 0.001$, A: $F(10,34) = 2.45$, $p = 0.025$]. ANOVA on the NA data only disclosed a significant main effect of time, $F(5,16) = 3.27$, $p < 0.05$. *Significantly different from corresponding vehicle/saline- and flumazenil/drug-treated group values. †Significantly different from corresponding vehicle/CDP group value.

According to the present results, it seems that occupation of central BDZ receptors either by agonists or inverse agonists produces qualitatively similar effects on the pituitary-adrenocortical axis, despite their opposite biochemical/electrophysiological intrinsic actions at the GABA-gated chloride ionophore. Thus it is clear that measurement of basal CS concentrations alone cannot be used as a hormonal predictor of agonist- or inverse agonist-like actions at the level of the BDZ/GABA receptor chloride ionophore com-

plex. Determination of plasma NA and A levels may prove to be better hormonal indices in this respect.

In summary, this study shows that the CNS pathways regulating pituitary-adrenocortical and sympatho-adrenal output are functionally subserved by central-type BDZ receptors. Whether these receptors have a physiological role in the absence of an exogenous ligand is still a matter of considerable speculation. Final proof awaits the discovery of its putative endogenous ligand(s).

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